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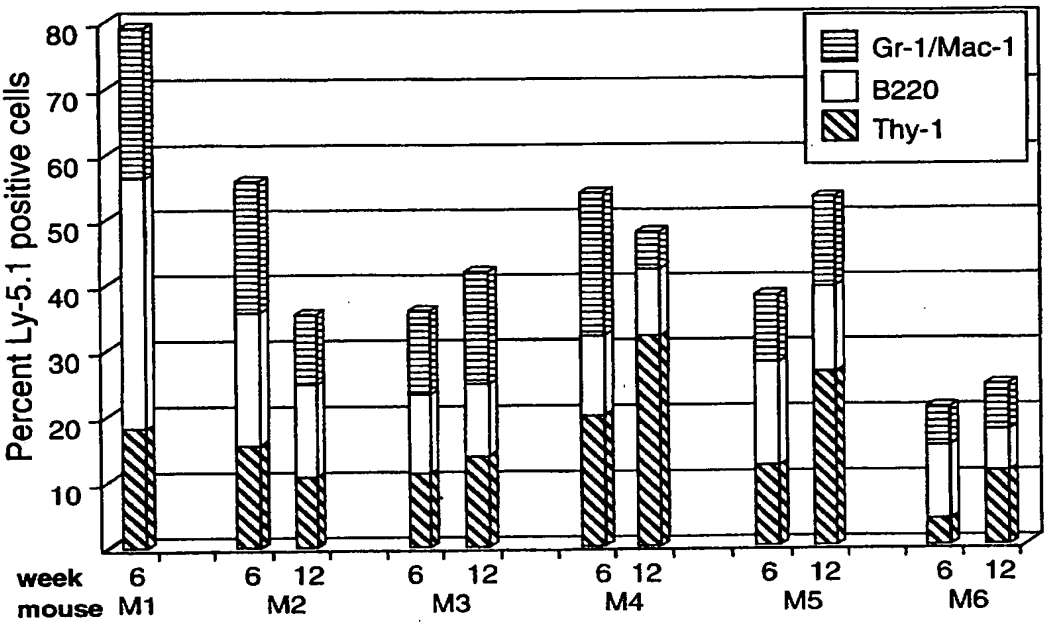
(54) Title: STEM CELLS DERIVED FROM SKELETAL MUSCLE



(57) Abstract: Materials and methods are provided related to a novel discovery that muscle stem cells (also called satellite cells) can generate at least the major hematopoietic lineages in individual mice over time as shown in the figure and the cells retain their regenerative potential after they are transferred to secondary recipients.

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APPLICATION FOR PATENT

STEM CELLS DERIVED FROM SKELETAL MUSCLE

SPECIFICATION

This invention claims priority to a United States provisional patent application Serial No. 60/141,206 filed June 25, 1999.

Field of the Invention

The present invention relates to the discovery of novel properties and uses for stem cells derived from skeletal muscle. More particularly, the present invention relates to materials and methods for regenerating various cell types in animals with the use of muscle stem cells. The materials and methods are related to the novel discovery that muscle stem cells, also called satellite cells, when removed from their *in situ* sites can differentiate into other cell types, such as hematopoietic cells.

Background of the Invention

Bone marrow transplantation is increasingly used in, for example, the treatment of hematologic malignancy, breast cancer, and other metabolic disorders. A key feature of bone marrow transplantation is the ability of transplanted hematopoietic stem cells to generate all the lineages of the blood of the transplant recipient over a period of time.

Bone marrow cells are also presently a focus of interest for gene therapy, due to the possibility that genetic modification of the stem cell can be manifested in downstream progeny. However, while hematopoietic stem cells can be transduced,

the efficiency of gene transfer is currently too low to allow realistic hope of therapeutic benefit from stem cell therapy using hematopoietic stem cells.

Another serious limitation in the use of bone marrow transplantation is that, for example, in autologous bone marrow transplantation, a patient's bone marrow may be contaminated with transformed cells that are difficult to remove from the bone marrow cells. For example, many leukemia cells cannot be separated from the bone marrow stem cells.

As an alternative to bone marrow transplantation, the use of human embryonic stem cells has been considered. However, because these cells must be derived from aborted human fetuses, there is substantial ethical controversy surrounding this technology (Shamblott, *et al.*, 1998; Thomson, *et al.*, 1998).

A major requirement of hematopoietic stem cell biology is that true stem cells must be highly proliferative and able to generate progeny that can repopulate secondary recipients (Siminovitch, *et al.*, 1963; and Spangrude, *et al.* 1991), although this property has well established limits (Jones, *et al.* 1989; and Mauch, *et al.*, 1989).

It is known that regenerative stem cells can be found in many adult tissues (Keller, *et al.*, 1992; Gage, *et al.*, 1995; Rudland, P. S., 1987; Sigal, *et al.*, 1992; Watt, F. M., 1998; and Schultz, *et al.*, 1994). Although possessing substantial proliferative and differentiative capacity, such cells are thought to be committed to differentiate exclusively into the tissue in which they reside. The myogenic potential of satellite cells is well established (Allbrook, D., 1981; Bischoff, R., 1986; Mauro, A., 1961; Snow, M. H., 1977). However, there have been some recent reports indicating multilineage potential for ostensibly tissue-specific progenitor cells (Bjornson, *et al.*, 1999; Ferrari, *et al.*, 1998; Horwitz, *et al.*, 1999; and Petersen, *et al.*, 1999). Moreover, it has recently been shown that neuronal stem cells have hematopoietic potential. Bjornson *et al.*, 1999. Also, for example, U.S. Patent Number 5,851,832 discloses methods for the *in vitro* proliferation and differentiation of neural stem cells and stem cell progeny.

The limitations mentioned above in using hematopoietic stem cells in gene therapy (the inefficiency of transformation and low efficiency of gene transfer) have been addressed with attempts to use different stem cells as gene therapy vectors. For example, U.S. Patent Number 5,750,376 discloses methods of producing genetically modified neuronal cells by transforming neuronal stem cells.

However, there remains in the art a need for stem cells that are not bone marrow or embryo derived, are not likely to be contaminated with transformed cells, and have the ability to differentiate in the body into various cell types, including all blood cell types. Further, it is desired in the art that such cells be readily and stablely transformable allowing for their use in gene therapy.

Summary of the Invention

An object of the invention is a method of replacing blood cells in an animal comprising the steps of administering muscle stem cells to an animal, wherein the muscle stem cells differentiate in the animal into blood cells.

An object of the invention additionally is a method of replacing bone marrow stem cells in an animal, comprising the steps of administering muscle stem cells to an animal, wherein the muscle stem cells are grafted to the bone marrow and/or home to the bone marrow in the animal, and differentiate in the animal into blood cells.

An object of the invention additionally is a method of replacing a particular differentiated cell type in an animal, comprising the steps of administering muscle stem cells to an animal, wherein the muscle stem cells differentiate in the animal into a particular differentiated cell type.

An object of the invention additionally is a method of replacing muscle cells in an animal, comprising the steps of administering muscle stem cells to an animal, wherein the muscle stem cells differentiate in the animal into muscle cells.

An object of the invention additionally is a method of gene therapy, comprising the steps of transducing muscle stem cells with nucleic acid, thereby

creating transduced muscle stem cells, and administering the transduced muscle stem cells to an animal.

Thus, in accomplishing the foregoing objectives, there is provided in accordance with one aspect of the present invention a method of replacing blood cells in an animal comprising the steps of administering muscle stem cells to an animal, wherein the muscle stem cells differentiate in the animal into blood cells. The method may further comprise the step of isolating muscle stem cells from skeletal muscle prior to administering the cells to the animal. Further, the muscle stem cells may be isolated from the same animal, or a different animal, as is administered the muscle stem cells. Still further, the muscle stem cells administered to the animal may be transduced muscle stem cells, which may be transduced after being isolated from skeletal muscle but prior to being administered to the animal. The animal may be a human.

In another embodiment of the present invention there is provided a method of replacing bone marrow stem cells in an animal, comprising the steps of administering muscle stem cells to an animal, wherein the muscle stem cells are grafted to the bone marrow and/or home to the bone marrow in the animal, and differentiate in the animal into blood cells. The method may further comprise the step of isolating muscle stem cells from skeletal muscle prior to administering the cells to the animal. Further still, the muscle stem cells may be isolated from the same animal, or from a different animal, as is administered the muscle stem cells. The muscle stem cells administered to the animal may also be transduced muscle stem cells, which may be transduced after being isolated from skeletal muscle and prior to being administered to the animal. The animal may be a human.

In another embodiment of the present invention there is provided a method of replacing a particular differentiated cell type in an animal, comprising the steps of administering muscle stem cells to an animal, wherein the muscle stem cells differentiate in the animal into a particular differentiated cell type, or multiple cell types. The method may further comprise the step of isolating muscle stem cells from

skeletal muscle prior to administering the cells to the animal. The muscle stem cells may be isolated from the same animal, or from a different animal, as is administered the muscle stem cells. The muscle stem cells may be transduced muscle stem cells, and may be transduced after being isolated from skeletal muscle and prior to being administered to the animal. The particular differentiated cell type may be a neuronal cell, an endothelial cell, or a muscle cell. The animal may be a human.

In another embodiment of the present invention there is provided a method of replacing muscle cells in an animal, comprising the steps of administering muscle stem cells to an animal, wherein the muscle stem cells differentiate in the animal into muscle cells. The method may further comprise the step of isolating muscle stem cells from skeletal muscle prior to administering the cells to the animal. The muscle stem cells may be isolated from the same animal, or a different animal, as is administered the muscle stem cells. Further, the muscle stem cells administered to the animal can be transduced muscle stem cells, which may be transduced after being isolated from skeletal muscle and prior to being administered to the animal. The animal may be a human.

Finally, in another embodiment of the present invention there is provided a method of gene therapy, comprising the steps of transducing muscle stem cells with nucleic acid, thereby creating a transduced muscle stem cells, followed by administering the transduced muscle stem cells to an animal. In the animal, the transduced muscle stem cells may differentiate into a differentiated cell type which can include a blood cell type, a neuronal cell, a muscle cell, or an endothelial cell. The muscle stem cells can be transduced using retroviral-mediated gene transfer. And, the animal may be a human.

Brief Description of the Drawings

Figures 1A, 1B, and 1C show analysis of peripheral blood following transplantation with muscle stem cells.

Figure 2 shows the percentage of Ly-5.1+ cells in individual mice over time.

Figure 3 shows the percentage of Ly-5.1+ cells.

Figures 4A - 4D analyze the percentages of cells with stem cell phenotype, and Ly-5.1+ phenotype.

The drawings are not necessarily to scale, and certain features of the invention may be exaggerated in scale or shown in schematic form in the interest of clarity and conciseness.

Detailed Description of the Preferred Embodiment

It is readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

The term "gene therapy" as used herein, refers to all methods of gene therapy whereby genetic information is utilized to change and/or supplement the genetic makeup of an animal, frequently for therapeutic purposes. This includes, but is not limited to, the use of recombinant DNA technology to deliver new and/or altered genetic sequences to an animal, including, for example, the use of a recombinant retrovirus to carry a particular genetic sequence.

The terms "muscle stem cell" and "muscle satellite cell" are used synonymously herein to denote what is known and understood in the art as muscle stem cells, also called muscle satellite cells, briefly, cells found surrounding muscle fibers that have been, for example, shown to be involved in muscle regeneration after injury. Such cells can be obtained from skeletal muscle of any age, although their prevalence is higher in younger individuals (Snow, M. H, 1977; Campion, D. R., 1984).

The term "blood cell(s)" as used herein denotes any cells known in the art to be blood cells. This includes, but may not be limited to, cells of lymphoid and myeloid lineages, B cells, T cells and granulocytes/macrophages.

The term "isolating" as used herein for example in the context of "isolating muscle stem cells" and the like refers to isolation as known in the art. For example,

in the case of isolating muscle stem cells from muscle, it denotes any separation and/or segregation of muscle stem cells from muscle tissue regardless of the degree of separation or processes and/or methods used. Furthermore, it is to be noted that in cases in which autologous bone marrow is impaired (as in aplastic anemia) or transplantation is required for cancer therapy but the marrow is likely to be contaminated by tumor cells (as in neuroblastoma), muscle satellite cells can be isolated to be free, or essentially free, or contaminating tumor cells, thereby providing a tumor-cell free (or lean) source of hematopoietic stem cells.

The terms "transduce(d)" and "transform(ed)" as used herein are used as understood in the art, and are used broadly herein to cover any introduction of, and result of, genetic material (regardless of its nature, e.g., DNA, RNA, viral, etc.) by any means (e.g., mechanical, chemical, viral, electromagnetic, homologous recombination, gene replacement technology, and so forth) into a cell or tissue to create a cell or tissue having (permanently or transiently, expressed or non-expressed) introduced material capable of expressing or encoding a genetic sequence.

The following detailed examples of the preferred embodiment relate to an illustrative examples of the present invention. It is to be understood that these examples are in no way intended to limit the scope of the present invention but merely illustrate examples of preferred embodiments presently known to the inventors. Additional embodiments are within the scope of the present invention.

It has been discovered, for example, that muscle stem cells, when removed from muscle and administered intravenously, home to the bone marrow and give rise to all of the differentiated lineages of the adult blood system. This gives rise to materials and methods for use in regenerating cell lineages in animals, including uses in restorative therapy, treatment, and gene therapy. In at least one embodiment of the practice of the present invention, it has been discovered that muscle stem cells are more potent at generating blood cells than are transplanted bone marrow cells.

In practicing embodiments of the present invention, various approaches may be taken, including the following non-limiting examples. It is understood that those

skilled in the art may contemplate varieties and additional examples and methods within the scope of the present and claimed invention.

One aspect of the invention provides a method of replacing blood cells in an animal comprising the step of administering muscle stem cells to an animal, wherein the muscle stem cells differentiate in the animal into blood cells. The method may further comprise the step of isolating muscle stem cells from skeletal muscle prior to administering the cells to the animal. The muscle stem cells may be isolated from the same animal, or a different animal, as is administered the muscle stem cells. Also in this method, the muscle stem cells that are administered to the animal may be transduced muscle stem cells. These cells may find use in methods of gene therapy as will be understood by one skilled in the art. This method is applicable to animals including humans.

Another example of the practice of an embodiment of the present invention relates to a method of replacing bone marrow stem cells in an animal, comprising the step of administering muscle stem cells to an animal, wherein the muscle stem cells home to the bone marrow in the animal and differentiate in the animal into blood cells. The method may further comprise the step of isolating muscle stem cells from skeletal muscle prior to administering the cells to the animal. The muscle stem cells may be isolated from the same animal, or a different animal, as is administered the muscle stem cells. Further, in the method the stem cells may be transduced stem cells, thereby providing, for example, gene therapy use as is understood in the art. This method has application in animals including humans.

The practice of still another embodiment of the present invention provides a method of replacing a particular differentiated cell type in an animal, comprising the steps of administering muscle stem cells to an animal, wherein the muscle stem cells differentiate in the animal into a particular differentiated cell type. In this method, the stem cells may be targeted to given locations in order for the cells to differentiate into particular cell types. One skilled in the art will understand a number of methods are possible for targeting the administered muscle stem cells to differentiate in the

animal body into particular cell types and/or in particular locations. For example, the muscle target cells may be targeted by implanting them into select tissue sites in the animal. For example, where blood cells are the desired progeny of the stem cells, the muscle stem cells may be grafted directly into bone marrow in the animal. It also understood that intravenously administered muscle stem cells will home to the bone marrow to establish and produce differentiated blood cells. Additionally, the muscle stem cells can be targeted by injecting them and/or otherwise implanting them into particular tissues of interest, such as muscle and neuronal tissues. Other means of targeting cells are also within the scope of the invention, such as the use of targeting antibodies, cell receptors, viral means, genetically engineered means and so forth. The practice of this method may also comprise the step of isolating muscle stem cells from skeletal muscle prior to administering the cells to the animal. The muscle stem cells may be isolated from the same, or a different, animal as is administered the muscle stem cells. The muscle stem cells administered to the animal may be transduced muscle stem cells which may be used in gene therapy methods as understood in the art. Further still, the particular differentiated cell types may be, for example, blood cells, including cells of lymphoid lineages, cells of myeloid lineages, B cells, T cells, and granulocytes/macrophages, neuronal cells, endothelial cells, and for example, muscle cell. It is further understood that in the practice of this and other related methods, the muscle stem cells may be caused to differentiate into particular progeny cell types by applying various chemical and/or physical signals known in the art to cause stem cells to differentiate into particular cell types. This can include targeting the stem cells to particular locations in the body, as discussed above, and/or applying various chemical and/or physicals signals both in the body and *in vitro* prior to administering the cells. These methods are known to those skilled in the art and include, for example, the techniques set forth in U.S. Patent Numbers 5,750,376, 5,851,832, and 5,817,773. The animals used in this method may include humans.

It is further disclosed and understood by those skilled in the art that various embodiments of the practice of the present invention result in the differentiation of

muscle stem cells into a number of different types of differentiated cell types, including but not limited to skeletal muscle, cardiac muscle, hematopoietic cells, including red blood cells, platelets, T cells, B cells, granulocytes, macrophages, and other blood cells, neurons, glial cells, endothelial cells, smooth muscle cells, epithelial cells, including those of mammary gland, testes, gastrointestinal tract and liver, adipocytes, cartliage, bone, stromal cells of bone marrow, thymus, spleen, mesenchymal cells of other tissues, including liver, uterus, kidney, germ cells that may produce eggs or sperms, and pancreatic cells.

Other embodiments of the practice of the present invention include methods of method of replacing muscle cells in an animal, comprising the step of administering muscle stem cells to an animal, wherein the muscle stem cells differentiate in the animal into muscle cells. This method may further comprise the step of isolating muscle stem cells from skeletal muscle prior to administering the cells to the animal. In the practice of this method, the muscle stem cells may be isolated from the same animal, or a different animal, as is administered the muscle stem cells. Further, for use in, for example, gene therapies, the muscle stem cells can be transduced muscle stem cells. In this method the animal may be a human or other animal.

Another embodiment of the practice of the present invention involves a method of gene therapy, comprising the steps of transducing muscle stem cells with nucleic acid, thereby creating transduced muscle stem cells, and administering the transduced muscle stem cells to an animal. This method may further include the transduced muscle stem cells differentiating in the animal into a differentiated cell type which may include a blood cell type, a neuronal cell, a muscle cell, an endothelial cell, or any number of different types of cells that differentiate from muscle stem cells. In this, and other gene-therapy related methods, the muscle stem cells may be transduced using retroviral-mediated gene transfer, or homologous recombination and/or gene replacement therapy and other techniques known in the art. The animal may be a human.

Gene Therapy Administration

The method of cell therapy may be employed by methods known in the art wherein a cultured cell containing a copy of a nucleic acid sequence or amino acid sequence for therapy of cancer is introduced.

In an embodiment of the present invention the cells and methods of the present invention are utilized for gene therapy. For gene therapy, a skilled artisan would be cognizant that the cell contains a vector wherein the gene of interest is operatively limited to a promoter. For antisense gene therapy, the antisense sequence of the gene of interest would be operatively linked to a promoter. The promoter may be consitutive, inducible or tissue-specific. One skilled in the art recognizes that in certain instances other sequences such as a 3' UTR regulatory sequence is useful in expressing the gene of interest. Means known in the art can be utilized to prevent release and absorption of the composition until it reaches the target organ or to ensure timed-release of the composition. A sufficient amount of vector containing the therapeutic nucleic acid sequence is administered to provide a pharmacologically effective dose of the gene product.

One skilled in the art recognizes that different methods of delivery may be utilized to administer a vector into a cell of the present invention. Examples include: (1) methods utilizing physical means, such as electroporation (electricity), a gene gun (physical force) or applying large volumes of a liquid (pressure); and (2) methods wherein said vector is complexed to another entity, such as a liposome or transporter molecule.

Accordingly, the present invention provides a method of transferring a therapeutic gene to a host, which comprises administering the vector inside a cell of the present invention. Effective gene transfer of a vector to a host cell in accordance with the present invention can be monitored in terms of a therapeutic effect (e.g. alleviation of some symptom associated with the particular medical condition being treated) or, further, by evidence of the transferred gene or expression of the gene within the host (e.g., using the polymerase chain reaction in conjunction with

sequencing, Northern or Southern hybridizations, or transcription assays to detect the nucleic acid in host cells, or using immunoblot analysis, antibody-mediated detection, mRNA or protein half-life studies, or particularized assays to detect protein or polypeptide encoded by the transferred nucleic acid, or impacted in level or function due to such transfer).

These methods described herein are by no means all-inclusive, and further methods to suit the specific application will be apparent to the ordinary skilled artisan. Moreover, the effective amount of the compositions can be further approximated through analogy to compounds known to exert the desired effect.

Furthermore, the actual dose and schedule can vary depending on whether the cells are administered in combination with other pharmaceutical compositions, or depending on interindividual differences in pharmacokinetics, drug disposition, and metabolism. Similarly, amounts can vary in *in vitro* applications depending on the particular cells utilized. Furthermore, the amount of vector to be added per cell will likely vary with the length and stability of the therapeutic gene inserted in the vector, as well as also the nature of the sequence, and is particularly a parameter which needs to be determined empirically, and can be altered due to factors not inherent to the methods of the present invention (for instance, the cost associated with synthesis). One skilled in the art can easily make any necessary adjustments in accordance with the exigencies of the particular situation.

It is possible that cells containing the therapeutic gene may also contain a suicide gene (i.e., a gene which encodes a product that can be used to destroy the cell, such as herpes simplex virus thymidine kinase). In some gene therapy situations, it is desirable to be able to express a gene for therapeutic purposes in a host cell but also to have the capacity to destroy the host cell once the therapy is completed, becomes uncontrollable, or does not lead to a predictable or desirable result. Thus, expression of the therapeutic gene in a host cell can be driven by a promoter although the product of said suicide gene remains harmless in the absence of a prodrug. Once the therapy is complete or no longer desired or needed, administration of a prodrug

causes the suicide gene product to become lethal to the cell. Examples of suicide gene/prodrug combinations which may be used are Herpes Simplex Virus-thymidine kinase (HSV-tk) and ganciclovir, acyclovir or FIAU; oxidoreductase and cycloheximide; cytosine deaminase and 5-fluorocytosine; thymidine kinase thymidilate kinase (Tdk::Tmk) and AZT; and deoxycytidine kinase and cytosine arabinoside.

In a specific embodiment the nucleic acid for therapy is a DNA or a RNA, and it is within the scope of the present invention to include any nucleic acid for a therapeutic purpose within the cells. Specific examples include but are not limited to the dystrophin nucleic acid, such as for the treatment of muscular dystrophy, or the beta-globin gene, such as for the treatment of sickle cell anemia. Other nucleic acids include ras, myc, raf, erb, src, fms, jun, trk, ret, gsp, hst, bcl abl, Rb, CFTR, p16, p21, p27, p53, p57, p73, C-CAM, APC, CTS-1, zac1, scFV ras, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, MMAC1, FCC, MCC, BRCA2, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 IL-12, GM-CSF G-CSF and thymidine kinase.

In a specific embodiment the nucleic acid for therapy is p53, which is often mutated in cancer. Alternatively, as is taught by Foster et al. (1999), herein incorporated by reference, a compound to stabilize the DNA binding domain of p53 in an active conformation is furthermore delivered via cells or methods of the present invention to enable a mutant p53 in a tumor cell to activate transcription and slow tumor growth. In a specific embodiment the compound for stabilization comprises a hydrophobic group containing at least one cyclic group joined by a linker to an ionizable group, such as an amine.

The following is an illustration of a preferred embodiments for practicing the present invention. However, they are not limiting examples. Other examples and methods are possible in practicing the present invention.

Example 1

Method of isolating satellite cells

The gastrocneimius, soleus, and plantaris were dissected from three C57Bl/6-Ly-5.1, 6-week old mice. Tendons, all bone, and fat were carefully discarded, the muscle tissue was thoroughly minced and then digested at 37° C with 0.2% collagenase (Worthington) for 45 minutes, followed by 0.1% trypsin (Gibco) for 45 minutes. The tissue was triturated vigorously, passed through a 70 μm filter, and the cells were collected by centrifugation. Cells were plated in Dulbecco's modified Eagle's medium (DME) containing 10% fetal calf serum (Hyclone), 5% chick embryo extract (Gibco), and antibiotics for 1 hour at 37° C. The non-adherent cells were then transferred to another plate and the adherent cells (primarily fibroblasts) were discarded. After 24 hours, the floating cells (blood cells) and debris were vigorously washed off the plate, and fresh medium was applied to the attached satellite cells.

Example 2

Method of bone marrow transplantation

Satellite cells were harvested by trypsinization after 5 days of culture, counted, and mixed with 200 x 10^5 nucleated whole bone marrow cells prepared from 6- to 12-week old C57Bl/6-Ly-5.2 mice. Recipients were also 6- to 12-week old C57Bl/6-Ly-5.2 mice that had been given 11 Gy in a split dose and maintained on acidified water and autoclaved food. Cell mixtures were injected retroorbitally in a volume of 300 μl under methoxyflurane anesthesia (Goodell, *et al.*, 1996; Goodell, *et al.*, 1997). For the secondary transplant, bone marrow was harvested from mouse 1, and 8 x 10^5 nucleated cells were injected into five C57Bl/6-Ly-5.2 recipients prepared as described herein. It is noted that in another embodiment, intravenous administration of muscle stem cells results in their homing to the bone marrow, providing, for example, another method for providing muscle stem cells in the bone marrow of an animal.

Example 3

Analysis of blood from transplant recipients

Six and 12 weeks after transplantation, 150 µl of peripheral blood was collected from the retroorbital plexus under methoxyflurane anesthesia. The nucleated cells were then stained with anti-Ly-5.1-biotin (clone A20), B220-FITC (RA3-6B2), Thy-1-FITC (30-H12), Gr-1-FITC (RB6-8C5) and Mac-1-FITC (M1/70) (all from Pharmingen). Ly-5.1-biotin was detected by a subsequent staining with streptavidin-phycoerythrin (Molecular Probes). The stained blood samples were analyzed by flow cytometry on a FACSCalibur (Becton Dickinson). Background staining of Ly-5.2-positive peripheral blood by anti-Ly-5.1 antibody on was consistently less than 0.5%.

Example 4

Hematopoietic stem cell enumeration

Secondary transplant recipient mouse 5 was sacrificed and bone marrow was prepared and stained with Hoechst dye as known in the art (for example, Goodell, *et al.*, 1996; and Goodell, *et al.*, 1997) or downloaded, Goodell, M. A. http://www.bcm.tmc.edu/genetherapy/goodell/page2.htm. In brief, bone marrow was suspended at 10^6 nucleated cells per ml in DME with 2% fetal calf serum (Hyclone), 10 mM HEPES buffer (Gibco) and 5 µg/ml Hoechst 33342 (Sigma) and incubated at 37°C for 90 minutes. The bone marrow was pelleted by centrifugation and resuspended at 10^8 cells per ml in cold Hanks balanced salt solution containing 2% fetal calf serum and 10 mM HEPES (HBSS+) for staining with anti-Ly-5.1-biotin and streptavidin-PE on ice. The cells were resuspended in HBSS+ containing 2 µg per ml propidium iodide (Sigma). Flow cytometric analysis was performed on a triple-laser instrument (MoFlow, Cytomation, Inc.). An argon laser tuned to 350 nm emission was used to excite the Hoechst dye. Fluorescence emission was collected with a 405/30 BP filter (Hoechst blue) and 670/40 BP filter (Hoechst red). A second 488

nm argon laser was used to excite phycoerythrin (emission was collected with a 575/40 BP filter).

Example 5

Transplantation of murine satellite cells into the bone marrow of lethally irradiated mice

To transplant murine satellite cells into the bone marrow of lethally irradiated mice, a competitive bone marrow transplantation model was used in which the test cells are co-transplanted with whole bone marrow from a distinguishable strain of mice (Harrison, D. E., 1980). This material provides sufficient numbers of committed hematopoietic cells and stem cells to rescue the mice from lethal irradiation, allowing measurement of the stem cell activity of the test population against a known quantity of stem cells in the competitor population.

Satellite cells were prepared from C57Bl/6-Ly-5.1 mice, mixed with whole bone marrow from C57Bl/6-Ly-5.2 mice, and transplanted into lethally irradiated C57Bl/6-Ly-5.2 mice. Six weeks after transplantation, peripheral blood was drawn from the recipients and analyzed by antibody staining and flow cytometry for the presence of B cells (B220), T Cells (Thy-1) and granulocytes/macrophages (Gr-1+Mac-1) derived from the Ly-5.1+ satellite cells. The results of one representative experiment are shown in Fig. 1. In this animal, the total proportion of Ly-5.1+ cells in the peripheral blood was 56%. Such cells were present at high levels in each of the lymphoid and myeloid lineages (upper right quadrant of each panel). The proportions of B cells, T cells, and granulocytes/macrophages derived from satellite cells were 42%, 76%, and 52%, respectively. The overall distribution of B, T, and myeloid cells in this mouse was normal.

The mean percentage of satellite cell progeny in the peripheral blood of six mice at 6 weeks post-transplantation was 56% (SD, 20%). Since only 18 x 10^3 satellite cells were introduced, compared with 200 x 10^3 whole bone marrow cells, this result indicates that the satellite stem cell activity was approximately 14-fold

higher than that of whole bone marrow. Mouse 1 (Fig. 2), which had the highest level of Ly-5.1+ peripheral blood cells (79%) was sacrificed and its bone marrow transplanted into lethally irradiated C57Bl/6-Ly-5.2 mice for subsequent study (below). In the remaining five animals, the mean percentage of Ly-5.1+ cells was 49% (SD, 9%). Twelve weeks after transplantation, its was 53% (SD, 15%). Thus, despite changes in the prevalence of Ly-5.1+ cells in individual mice over time, the cells retained their multilineage potential and remained at high levels (Fig. 2), demonstrating the long-term regenerative capacity of satellite stem cells.

These results show, *inter alia*, that when delivered to lethally irradiated mice in bone marrow transplants, satellite cells were approximately ten fold more potent than whole bone marrow at generating lymphoid and myeloid progeny including B cells, T cells, granulocytes and macrophages for at least 3 months post-transplantation. These observations show that satellite cells surrounding adult muscle fibers have much greater differentiative potential than previously suspected.

Example 6

Transplantation of murine satellite cells into the bone marrow of lethally irradiated mice

At 5 weeks post-transplantation, bone marrow was collected from mouse 1 (79% Ly-5.1+ cells) and tested it for regenerative capacity in lethally irradiated C57Bl/6-Ly-5.2 mice. As shown in Fig. 3, bone marrow from this mouse rescued all five recipients from lethal irradiation and contributed appreciably to the B, T, and myeloid-cell compartments. The mean percentage of Ly-5.1+ cells in the peripheral blood of these mice was approximately 37% (SD, 23%). Notably, there was a shift in the distribution of blood cell lineages, marked by lower percentages of T and B cells than were found in the original recipients (Fig. 2). This may reflect the relatively short time post-transplantation or perhaps a reduction in long-term regenerative potential relative to that of the competing Ly-5.2+ bone marrow stem cells within the transplant.

To verify that Ly-5.1+ hematopoietic stem cells were present in the secondary transplants, mouse 5 was sacrificed (Fig. 3) and stained its bone marrow with the dye Hoechst 33342 and anti-Ly-5.1 antibody. Hematopoietic stem cells were identified on the basis of their high dye efflux activity, as determined by dual wavelength analysis of Hoechst dye fluorescence (Goodell, *et al.*, 1996; Goodell, *et al.*, 1997). As shown in Fig. 4A, approximately 0.03% of the whole bone marrow had a stem cell phenotype and 63% of the whole bone marrow was positive for the Ly-5.1 marker (Fig. 4B). Closer examination of the hematopoietic stem cell population (Fig. 4C) revealed that a majority of the cells (65%) were positive for the Ly-5.1 marker (Fig. 4D) and thus were derived from the satellite cells transplanted into the primary recipient, mouse 1.

These results show that at least some of these cells have hematopoietic potential. Since the prevalence of multipotent hematopoietic stem cells in bone marrow ranges from 0.05-0.01%, and the satellite cells in our study were approximately 10- to 14-fold more active than the competing bone marrow cells, we estimate that 0.7-0.1% of the Ly-5.1+ satellite cells were capable of generating hematopoietic precursors.

These results show, *inter alia*, that the cells retained their regenerative potential after they were transferred to secondary recipients, demonstrating their extremely primitive nature. These observations further confirm that satellite cells surrounding adult muscle fibers have much greater differentiative potential than previously suspected.

Example 7

Gene Therapy Example/Transplantation of transduced murine satellite cells

The cells and methods of the present invention are used in gene therapy methods, for example by transplanting transduced murine satellite cells (containing a genetic sequence of choice for use in gene therapy).

One method of practicing this embodiment of the present invention is to utilize retroviral-mediated gene transfer for vectoring a genetic sequence of choice into muscle stem cells prior to transplantation. Methods of gene therapy and well known in the art. In particular, but not in a limiting sense, it is noted that methods exist for the transduction of muscle stem cells. For example, U.S. Patent Number 5,466,676, which is hereby incorporated by reference in its entirety, provides methods of using retroviral-mediated gene transfer into muscle cells.

Such transduced cells are then administered to an animal as directed herein and as understood in the art, providing transgenic differentiated cells within the animal for gene therapy uses. For example, these techniques are used in gene therapy of hematopoietic disease and gene therapy of neuro-muscular disease.

It has been discovered and shown that adult muscle stem cells, called satellite cells, can generate at least the major hematopoietic lineages. When delivered to lethally irradiated mice in bone marrow transplants, satellite cells were approximately ten fold more potent than whole bone marrow at generating lymphoid and myeloid progeny including B cells, T cells, granulocytes and macrophages for at least 3 months post-transplantation. Moreover, the cells retained their regenerative potential after they were transferred to secondary recipients, demonstrating their extremely primitive nature. These observations indicate that satellite cells surrounding adult muscle fibers have much greater differentiative potential than previously suspected. Such cells have numerous medical applications including, for example, the provision of hematopoietic potential to patients in whom autologous bone marrow is impaired in function or contaminated by tumor, and gene therapy use in treatment of a number of disorders including, for example, hematopoietic and neuro-muscular diseases.

All patents and publications mentioned in this specification are indicative of levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication is specifically and individually indicated to be incorporated by reference.

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One skilled in the art will readily appreciate the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods, procedures, treatments, molecules and specific compounds described herein are presently representative of preferred embodiments, are exemplary and are not intended as limitations on the scope of the

invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention and are defined by the scope of the claims.

CLAIMS

What is claimed is:

1. A method of replacing blood cells in an animal comprising the step of:

administering muscle stem cells to an animal, wherein the muscle stem cells differentiate in the animal into blood cells.

2. The method of claim 1, further comprising the step of isolating muscle stem cells from skeletal muscle prior to administering the cells to the animal.

3. The method of claim 2, wherein the muscle stem cells are isolated from the same animal as is administered the muscle stem cells.

4. The method of claim 2, wherein the muscle stem cells are isolated from a different animal as is administered the muscle stem cells.

5. The method of claim 1, wherein the muscle stem cells are transduced muscle stem cells.

6. The method of claim 2, wherein the muscle stem cells administered to the animal are transduced muscle stem cells.

7. The method of claim 6, wherein the muscle stem cells are transduced after being isolated from skeletal muscle.

8. The method of claim 1, wherein the animal is a human.

9. A method of replacing bone marrow stem cells in an animal, comprising the step of:

administering muscle stem cells to an animal, wherein the muscle stem cells home to the bone marrow in the animal and differentiate in the animal into blood cells.

10. The method of claim 9, further comprising the step of isolating muscle stem cells from skeletal muscle prior to administering the cells to the animal.

11. The method of claim 10, wherein the muscle stem cells are isolated from the same animal as is administered the muscle stem cells.

12. The method of claim 10, wherein the muscle stem cells are isolated from a different animal as is administered the muscle stem cells.

13. The method of claim 9, wherein the muscle stem cells are transduced muscle stem cells.

14. The method of claim 10, wherein the muscle stem cells administered to the animal are transduced muscle stem cells.

15. The method of claim 14, wherein the muscle stem cells are transduced after being isolated from skeletal muscle.

16. The method of claim 9, wherein the animal is a human.

17. A method of replacing a particular differentiated cell type in an animal, comprising the steps of:

administering muscle stem cells to an animal, wherein the muscle stem cells differentiate in the animal into a particular differentiated cell type.

18. The method of claim 17, further comprising the step of isolating muscle stem cells from skeletal muscle prior to administering the cells to the animal.

19. The method of claim 17, wherein the muscle stem cells are isolated from the same animal as is administered the muscle stem cells.

20. The method of claim 17, wherein the muscle stem cells are isolated from a different animal as is administered the muscle stem cells.

21. The method of claim 17, wherein the muscle stem cells are transduced muscle stem cells.

22. The method of claim 18, wherein the muscle stem cells administered to the animal are transduced muscle stem cells.

23. The method of claim 22, wherein the muscle stem cells are transduced after being isolated from skeletal muscle.

24. The method of claim 17, wherein the particular differentiated cell type is a blood cell.

25. The method of claim 24, wherein the blood cell is selected from the group consisting of cells of lymphoid lineages, cells of myeloid lineages, B cells, T cells, and granulocytes/macrophages.

26. The method of claim 17, wherein the particular differentiated cell type is a neuronal cell.

27. The method of claim 17, wherein the particular differentiated cell type is an endothelial cell.

28. The method of claim 17, wherein the particular differentiated cell type is a muscle cell.

29. The method of claim 17, wherein the animal is a human.

30. A method of replacing muscle cells in an animal, comprising the step of:

administering muscle stem cells to an animal, wherein the muscle stem cells differentiate in the animal into muscle cells.

31. The method of claim 30, further comprising the step of isolating muscle stem cells from skeletal muscle prior to administering the cells to the animal.

32. The method of claim 30, wherein the muscle stem cells are isolated from the same animal as is administered the muscle stem cells.

33. The method of claim 30, wherein the muscle stem cells are isolated from a different animal as is administered the muscle stem cells.

34. The method of claim 30, wherein the muscle stem cells are transduced muscle stem cells.

35. The method of claim 31, wherein the muscle stem cells administered to the animal are transduced muscle stem cells.

36. The method of claim 35, wherein the muscle stem cells are transduced after being isolated from skeletal muscle.

37. The method of claim 30, wherein the animal is a human.

38. A method of gene therapy, comprising the steps of:

transducing muscle stem cells with nucleic acid, thereby creating transduced muscle stem cells; and

administering the transduced muscle stem cells to an animal.

39. The method of claim 38, further wherein the transduced muscle stem cells differentiate in the animal into a differentiated cell type.

40. The method of claim 39, wherein the differentiated cell type is selected from the group consisting of a blood cell type, a neuronal cell, a muscle cell, or an endothelial cell.

41. The method of claim 38, wherein the muscle stem cell is transduced using retroviral-mediated gene transfer.

42. The method of claim 38, wherein the animal is a human.

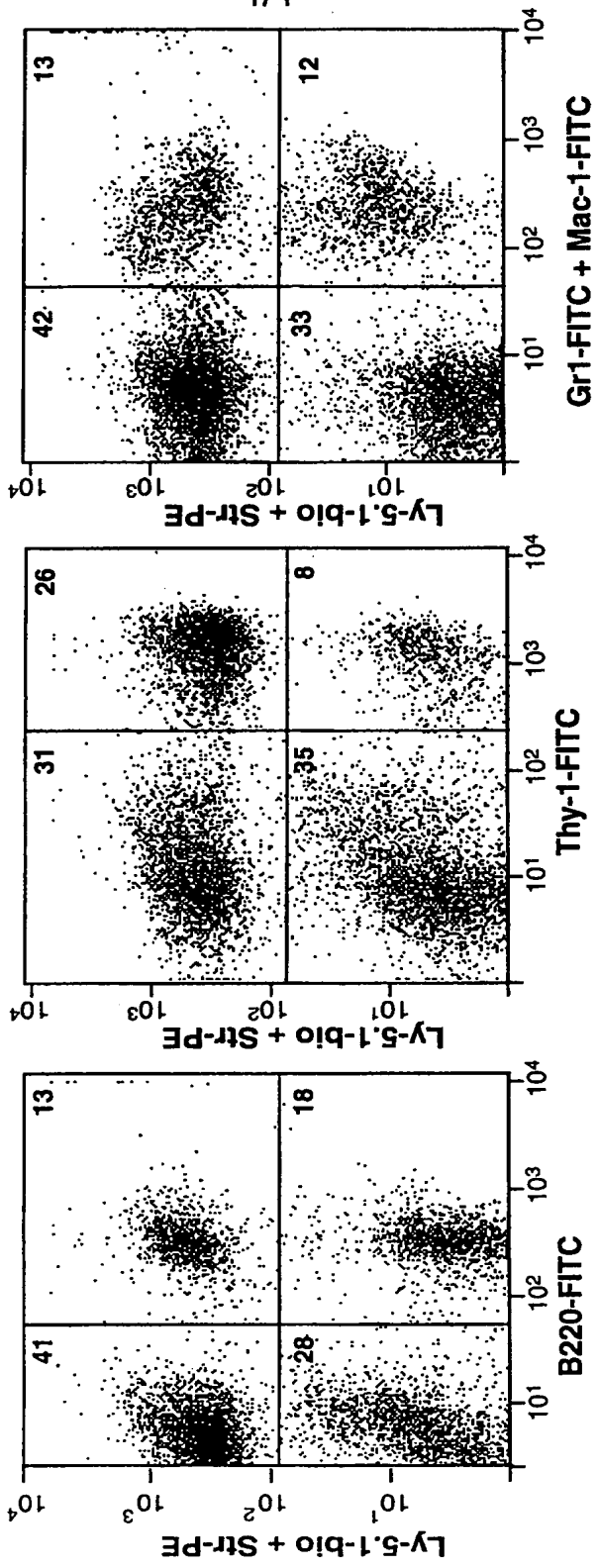

Figure 1C

Figure 1B

Figure 1A

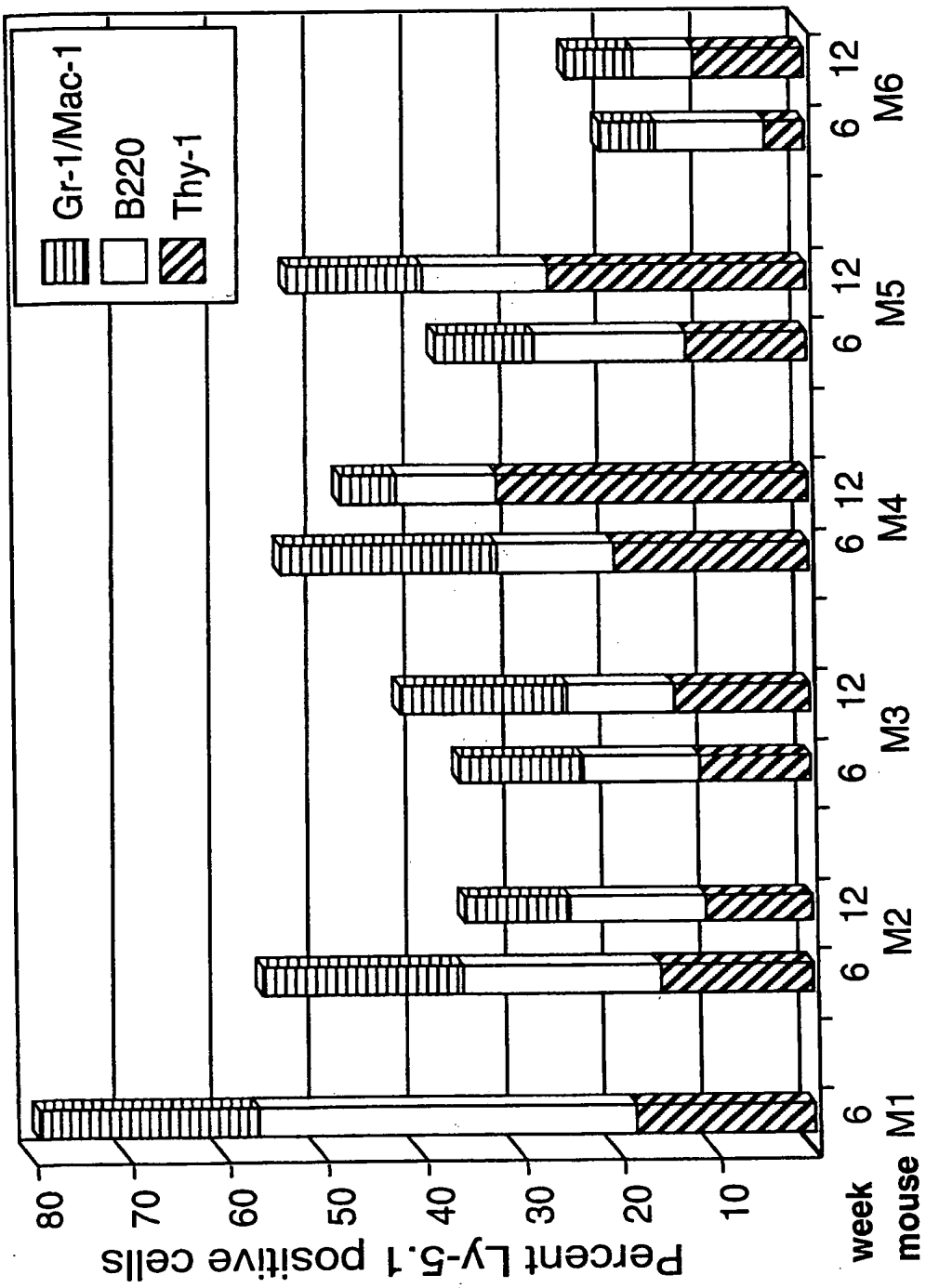

Figure 2

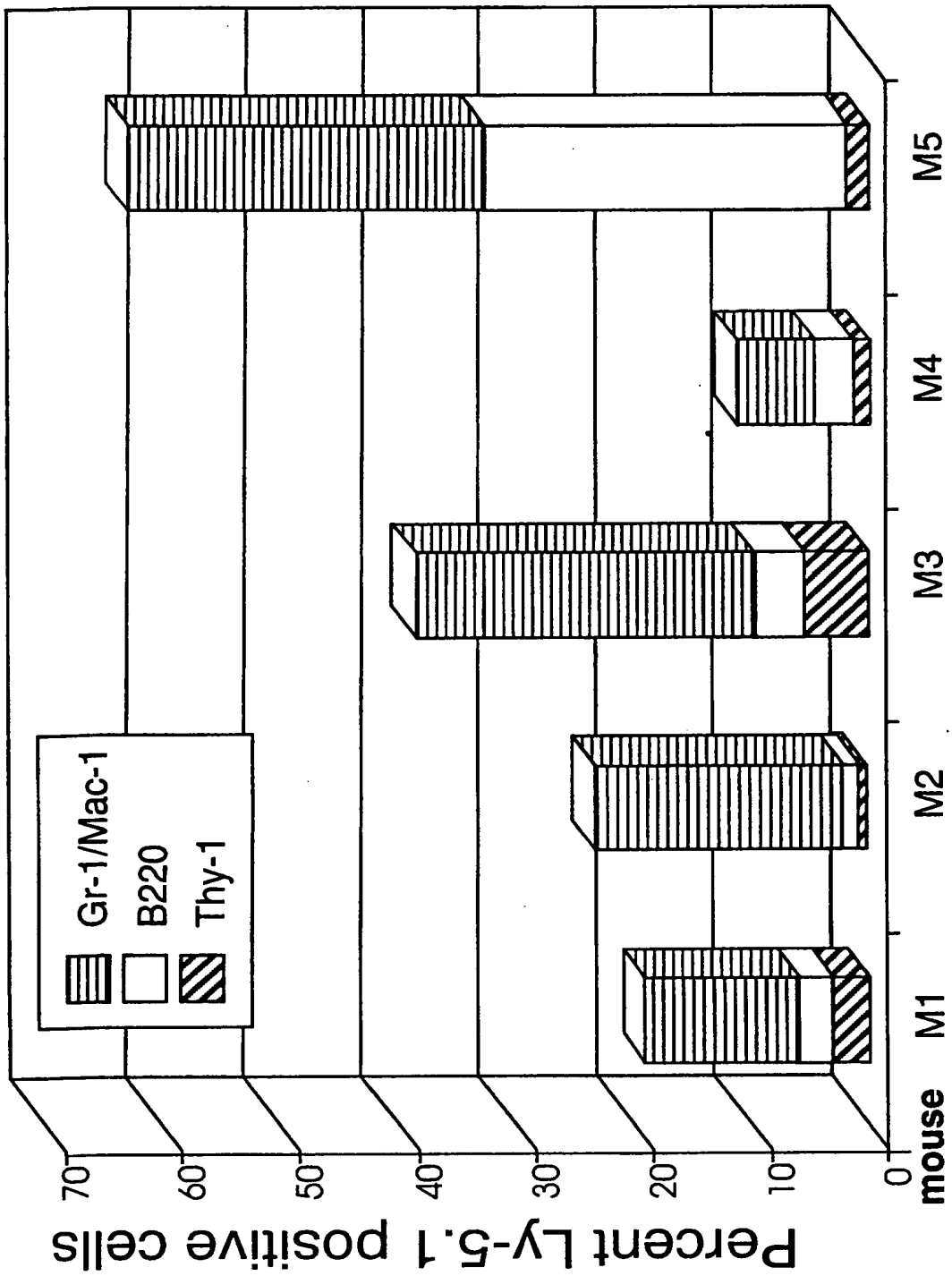

Figure 3

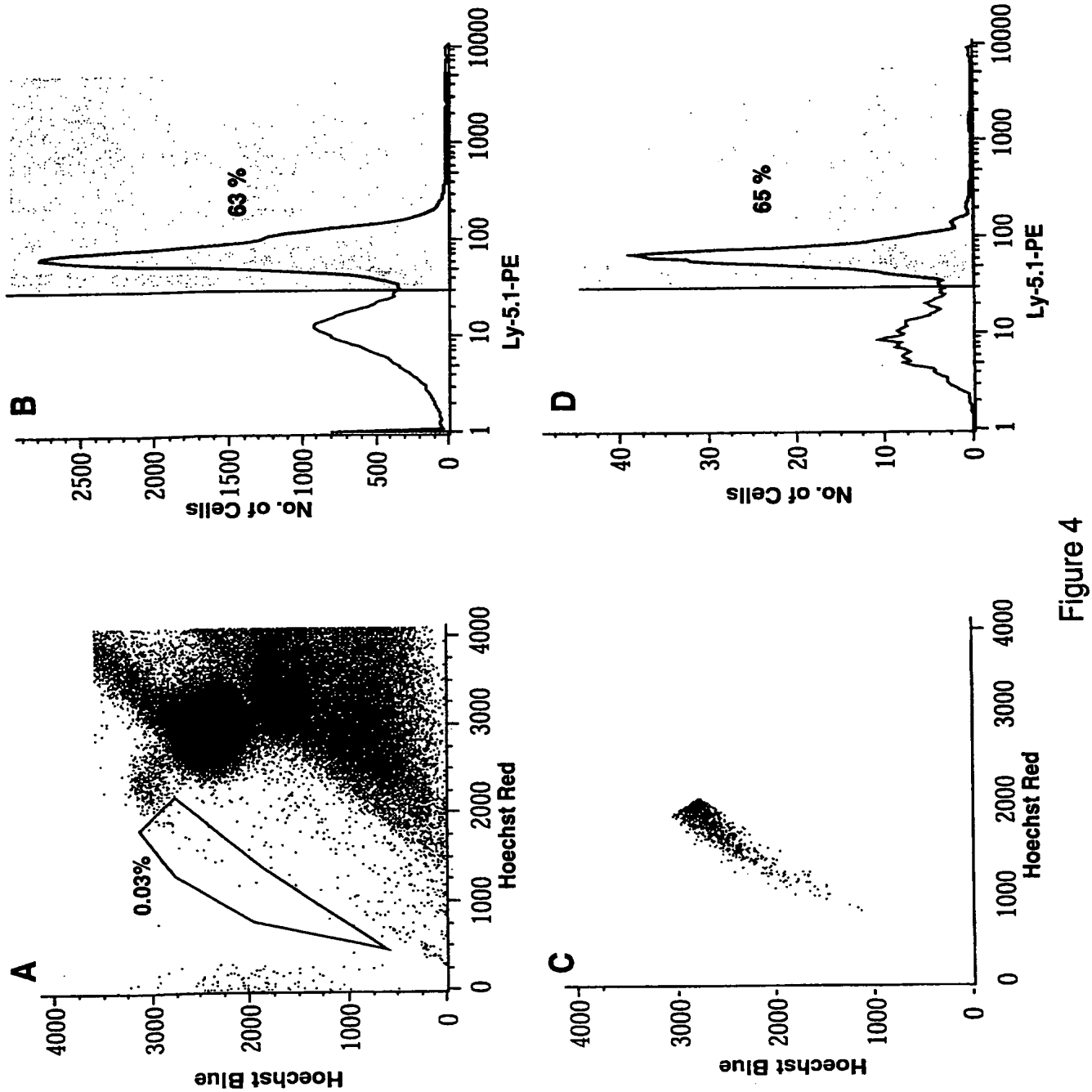

Figure 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/17064

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :A01N 63/00; C12N 5/00, 5/02, 15/86
US CL : 424/93.1, 93.21; 435/325, 456

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/93.1, 93.21; 435/325, 456

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ---- Y,P	JACKSON.K.A. et al. Hematopoietic potential of stem cells isolated from murine skeletal muscle. PNAS. 07 December 1999. Vol 96. No. 25. pages 14482-14486, see entire document, especially the abstract.	1,2,4,9,10, 12,17,18, 20,24,25 ---------- 3,5-8,11, 13-16,19, 21-23,28-42

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 26 JULY 2000	Date of mailing of the international search report 14 AUG 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer QUANG NGUYEN, PH.D. Telephone No. (703) 308-2801

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/17064

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P ---- Y, P	GUSSONI.E. et al. Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature. 23 September 1999. Vol 401. pages 390-394, see entire document, especially the abstract.	1,2,4,9,10, 12,17,18, 20,24,25, 28,30,31, 33 -------- 3,5-8,11, 13-16,19, 21-23,29, 32,34-42
X ----- Y	FERRARI.G. et al. Muscle regeneration by bone marrow-derived myogenic progenitors. Science. 06 March 1998. Vol. 279. pages 1528-1530, see entire document, especially Fig. 1 and column 3, first paragraph, page 1528.	17,18,20, 21,22,28, 30,31,33-35 ---------- 19,23,29, 32,36-42
X ----- Y	FERRARI.G. et al. A retroviral vector containing a muscle-specific enhancer drives gene expression only in differentiated muscle fibers. Hum. Gene Ther. June 1995. Vol 6. pages 733-742, see entire document, especially the abstract.	17,18,20-23,28, 30, 31,33-36 -------- 19,29,32, 37-42
X ---- Y	US 5,538,722 A (BLAU et al) 23 July 1996, see entire document, especially the summary.	17,18,20-23,28,30, 31,33-36 -------- 19,29,32, 37-42
A	BJORNSON.C.R.R. et al. Turning brain into blood: A hematopoietic fate adopted by adult neural stem cells in vivo. Science. 22 January 1999. Vol 283. pages 534-536, see entire document.	1-42

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/17064

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG, MEDLINE, BIOSIS, EMBASE
Search terms: stem cells, satellite cells, skeletal muscle, bone marrow, hematopoietic potential or fate, cell transplantation, cell differentiation